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Cathepsin D Specifically Cleaves the Chemokines Macrophage Inflammatory Protein-1α, Macrophage Inflammatory Protein-1β, and SLC That Are Expressed in Human Breast Cancer

Marlene Wolf,* Ian Clark-Lewis,† Caroline Buri,‡ Hanno Langen,§ Maddalena Lis,‡ and Luca Mazzucchelli‡

From the Theodor-Kocher Institute and the Institute of Pathology,‡ University of Bern, Bern, Switzerland; F. Hoffmann-La Roche Limited,§ Pharmaceutical Research Gene Technologies, Basel, Switzerland; and the Biomedical Research Center,† University of British Columbia, Vancouver, Canada*

Cathepsin D (Cath-D) expression in human primary breast cancer has been associated with a poor prognosis. In search of a better understanding of the Cath-D substrates possibly involved in cancer invasiveness and metastasis, we investigated the potential interactions between this protease and chemokines. Here we report that purified Cath-D, as well as culture supernatants from the human breast carcinoma cell lines MCF-7 and T47D, selectively degrade macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and SLC (CCL21). Proteolysis was totally blocked by the protease inhibitor pepstatin A, and specificity of Cath-D cleavage was demonstrated using a large chemokine panel. Whereas MIP-1α and MIP-1β degradation was rapid and complete, cleavage of SLC was slow and not complete. Mass spectrometry analysis showed that Cath-D cleaves the Leu^{58} to Trp^{59} bond of SLC producing two functionally inactive fragments. Analysis of Cath-D proteolysis of a series of monocyte chemoattractant protein-3/MIP-1β hybrids indicated that processing of MIP-1β might start by cleaving off amino acids located in the C-terminal domain. *In situ* hybridization studies revealed MIP-1α, MIP-1β, and Cath-D gene expression mainly in the stromal compartment of breast cancers whereas SLC transcripts were found in endothelial cells of capillaries and venules within the neoplastic tissues. Cath-D production in the breast carcinoma cell lines MCF-7 and T47D, as assessed by enzyme-linked immunosorbent assay of culture supernatants and cell lysates, was not affected by stimulation with chemokines such as interleukin-8 (CXCL8), SDF-1 (CXCL12), and SLC. These data suggest that inactivation of chemokines by Cath-D possibly influences regulatory mechanisms in the tumoral extracellular microenvironment that in turn may affect the generation of the antitumoral immune response, the migration of cancer cells, or both processes. *(Am J Pathol 2003, 162:1183–1190)*

Invasion and metastasis of cancer are the result of several pathogenic steps, including proliferation and detachment of neoplastic cells, breakdown of basement membrane, invasion of extracellular matrix, angiogenesis, movement across vascular barriers, and eventually, proliferation of cancer cells at new sites distant from the primary tumor. Numerous proteins have been shown to be involved in the regulation of cancer progression, but the underlying mechanisms at the molecular level remain primarily unidentified. In this context, proteolytic enzymes, ie, serine-, cysteine-, and aspartyl-containing proteases as well as matrix metalloproteases, which promote or directly contribute to the degradation and remodeling of the basement membrane and extracellular matrix, are considered of main importance.[1] For instance, numerous observations indicate that cathepsin D, one of the most widely studied proteases in cancer research, not only facilitates tumor progression[2] but may have a prognostic value in patients with breast cancer.[3] In recent years, the relationship between chemokines and malignant tumors has also emerged as an important research field. So far, most attention focused on the significance of chemokines in the recruitment of immune effector cells to a tumor site, in controlling cell growth and modulating the immune response, or in promoting angiogenesis during tumor progression.[4] Recent studies, however, indicate that chemokines and their receptors may directly affect the capacity of breast cancer cells to invade the extracellular matrix and that they may play a critical role in determining the metastatic destination of neoplastic cells.[5]

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Address reprint requests to Luca Mazzucchelli, M.D., Institute of Pathology, University of Bern, Murtenstrasse 31, 3010 Bern, Switzerland. E-mail: luca.mazzucchelli@ti.ch.

Very limited information is available on the interaction between chemokines and proteases. An increasing body of evidence indicates that chemokine effects may be terminated by chemokine degradation,[6–9] or that chemokines may directly contribute to the release of matrix remodeling enzymes.[10,11] We therefore investigated whether cathepsin D (Cath-D), which is highly expressed in human breast cancers, might degrade chemokines and consequently affect their putative functions in these tumors. Here, we report that macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and SLC (CCL21) are nonrandom substrates for Cath-D, and that all three chemokines are inactivated by this protease. In addition, we show that MIP-1α, MIP-1β, SLC, and Cath-D transcripts are detectable in close proximity within human breast cancers. The findings implicate novel regulatory mechanisms in the complex tumor microenvironment that affect the invasive capacity of breast cancer cells, the generation of tumor-specific immune responses, or both processes.

Materials and Methods

Cell Cultures, Reagents, and Tissue Specimens

The MCF-7 and T47D cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C in RPMI 1640 supplemented with penicillin, streptomycin, Glutamax, and 10% fetal calf serum. Human liver Cath-D and pepstatin A were purchased from Sigma Chemical Co., St. Louis, MO. Chemokines and chemokine hybrids were synthesized according to established protocols.[12] Formaldehyde-fixed and paraffin-embedded cancer specimens from 10 patients (mean age, 58 years) who underwent surgery for invasive ductal breast carcinoma were retrieved from the archives of the Institute of Pathology, University of Bern, Bern, Switzerland.

Chemokine Cleavage by Cath-D

Fifty nmol/L of Cath-D or 10-μl culture medium of MCF-7 or T47D cells were incubated with 2.5 to 5.0 μmol/L of chemokines at 37°C in 50 μl of a buffer containing 50 mmol/L sodium acetate and 50 mmol/L NaCl, pH 4.0. Parallel experiments were performed in the presence of the aspartate protease inhibitor pepstatin A. Reactions were stopped by heating to 90°C. The mixture was treated with 100 mmol/L of dithiothreitol for 15 minutes at 55°C, carboxymethylated with 200 mmol/L of iodoacetamide, and the chemokine cleavage products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10 to 20% Tris-Tricine polyacrylamide gels and stained with Coomassie blue. Mass determination of cleavage products was performed by nanoelectrospray mass spectrometry. Shortly, after cleavage with Cath-D, the chemokine samples were desalted and further concentrated on a pulled capillary containing ~100 nl of POROS R1 reverse phase material (Perceptive Biosystems, Framingham, MA). The peptides were eluted with 1 μl of 50% acetonitrile in 5% formic acid and 1 μl of 50% acetonitrile in 50% formic acid directly into the nanoelectrospray needle as described.[13] Electrospray mass spectra were acquired on an ESI QqTOF mass spectrometer (MDS Sciex, Concord, Ontario, Canada) equipped with a nanoelectronspray ion source developed by Wilm and Mann.[13] All data with a mass range from 800 to 2000 (m/z, amu) were acquired.

Purification and Characterization of Cath-D Cleavage Products

The two products obtained by Cath-D cleavage of SLC were purified by reverse phase column chromatography using a C2/C18 mRPC column (Amersham, Arlington Heights, IL) and elution with a linear gradient of 10 to 80% acetonitrile in 0.1% trifluoroacetic acid. Biological activity was assessed in CCR7-expressing mouse 300-19 pre-B cells[14] by measuring $[Ca^{2+}]_i$ changes according to standard protocols.[15] Because Cath-D completely degraded MIP-1α and MIP-1β, cleavage products of these chemokines were not available for analysis.

Chemokines and Cath-D Gene Expression

Probes for *in situ* hybridization were prepared as previously described.[16] Briefly, the following complementary human DNAs (cDNAs) were used: a 351-bp *Sma*I fragment of the MIP-1α cDNA, subcloned into pGEM-7 (Invitrogen, Groningen, The Netherlands); a 531-bp *Eco*RI fragment of MIP-1β cDNA subcloned into pBluescript SK+; an 828-bp *Eco*RI-*Not*I fragment of SLC cDNA subcloned into pT3T7; and a 2.4-kb *Eco*RI-*Xho*I fragment of Cath-D cDNA (a generous gift of Dr. P. Matthews, Orangenburg, NY), subcloned into a pcDNA3. After linearization with the appropriate restriction enzymes, sense and anti-sense probes were generated using SP6, T3, or T7 RNA polymerases (Roche Diagnostics, Basel, Switzerland) and ^{35}S-CTP (Amersham). The labeled probes were size-reduced by alkaline hydrolysis to an average length of 100 to 200 bases before precipitation. *In situ* hybridization of sections from formaldehyde-fixed and paraffin-embedded tissues was performed with minor modifications as previously described.[16] Tissue sections were dewaxed and rehydrated in graded ethanol. After treatment with 100 μg/ml of proteinase K (Roche Diagnostics) in 100 mmol/L of Tris-HCl, pH 8.0 and 50 mmol/L of ethylenediaminetetraacetic acid at 37°C for 30 minutes, tissues were hybridized with the indicated labeled sense or anti-sense probes overnight at 50°C in a moist chamber. Nonhybridized probe was removed by treatment with 20 μg/ml of RNase A and 1 U/ml of RNase T1 (Sigma Chemical Co.) and stringency washes. Slides were dipped into NTB-2 emulsion (Eastman-Kodak, New Haven, CT) diluted 1:2 in 800 mmol/L of ammonium acetate, pH 7.5. After exposure in the dark at 4°C for 4 weeks, slides were developed in Kodak PL-12 solution and counterstained with Gill's hematoxylin. Cells were considered to be positive for mRNA expression when they had at least three times as many silver grains as the

Table 1. Chemokine Processing by Cath-D (Respective Receptors in Brackets)

Processed by Cath-D	Not processed by Cath-D
MIP-1α (CCR1, CCR5)	IL-8, GCP-2 (CXCR1, CXCR2)
MIP-1β (CCR5)	Mig, IP-10, I-TAC (CXCR3)
SLC (CCR7)	SDF-1 (CXCR4)
	BCA-1 (CXCR5)
	RANTES (CCR1, CCR3, CCR5)
	MCP-1 (CCR2)
	MCP-2 (CCR3, CCR5)
	MCP-3 (CCR1, CCR2, CCR3)
	MCP-4 (CCR2, CCR3)
	Eotaxin, eotaxin-2 (CCR3)
	ELC (CCR7)
	I-309 (CCR8)

highest background obtained with the corresponding sense probe. Serial sections of all tissue samples were further studied by immunohistochemistry with commercially available antibodies directed against CD3 (T cells), and CD68 (macrophages). Immunohistochemistry was done using a standard streptavidin-biotin complex (ABC) technique with alkaline phosphatase as the enzyme and new fuchsin as the chromogen.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cath-D

MCF-7 and T47D cell lines were grown in 75-cm^2 flasks for 72 hours in culture medium alone or in the presence of 5 nmol/L of estradiol (Sigma Chemical Co.) and 125 ng/ml of epidermal growth factor (EGF, Sigma Chemical Co.). After harvesting, suspensions of 10^5 cells were starved in the same medium containing only 3% fetal calf serum and were allowed to adhere to wells of a microtiter plate for 18 hours at 37°C. Chemokines, including SLC, interleukin (IL)-8 (CXCL8), and SDF-1 (stromal cell-derived factor-1, CXCL12) were then added to duplicate wells at a final concentration of 100 nmol/L. After 24 hours of incubation at 37°C, the supernatants and the cell lysates were collected for enzyme assays. ELISA was performed with a commercially available kit (Oncogene, Boston, MA) according to the instructions provided by the manufacturer.

Results

Cath-D Degrades and Inactivates MIP-1α, MIP-1β, and SLC

A panel of 19 representative chemokines was incubated with purified Cath-D and processing was assessed by SDS-PAGE and staining with Coomassie blue. After 90 minutes of incubation at 37°C, MIP-1α, MIP-1β, and SLC, but not other chemokines, were degraded (Table 1). Time courses of chemokine cleavage (Figure 1) show that Cath-D completely degraded MIP-1α and MIP-1β. Cleavage products of these two chemokines could be detected only transiently by SDS-PAGE. Proteolysis of MIP-1β was detectable as early as 15 minutes after addition of Cath-D and completed within 1 hour, whereas total degradation of MIP-1α occurred after 2 hours. By contrast, SLC cleavage progressed more slowly, resulted in two distinct cleavage products, and was not completed after 4 hours. Incomplete SLC cleavage was even observed when aliquots of fresh Cath-D were added every hour to the reaction mixtures to overcome possible inactivation of the enzyme. Addition of aspartate protease inhibitor pepstatin A prevented proteolysis of all three chemokines. Chemokine cleavage was also abrogated by incubations at pH greater than 5.5.

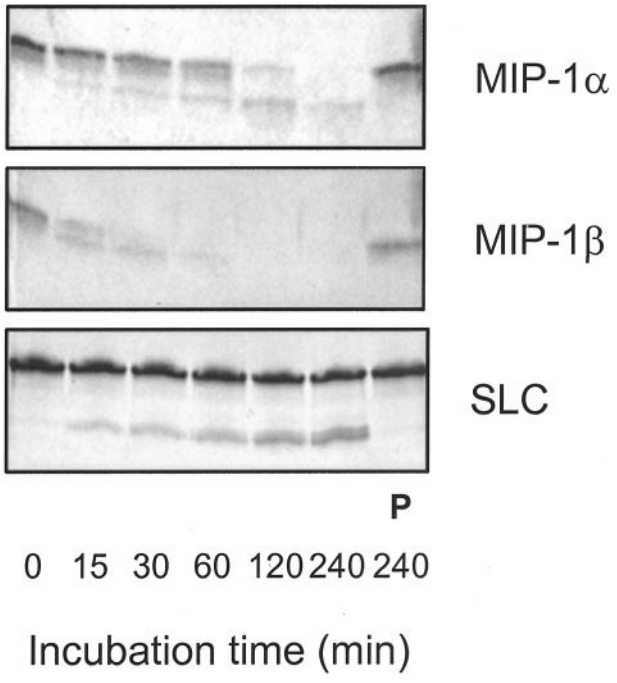

Figure 1. MIP-1α, MIP-1β, and SLC are substrates for Cath-D. Purified Cath-D was incubated with 2.5 μmol/L of MIP-1α, MIP-1β, or SLC at 37°C. Products were taken at the indicated times, separated by SDS-PAGE, and stained with Coomassie blue. Staining of MIP-1β was constantly weak. Addition of 1 μmol/L of aspartate protease inhibitor pepstatin A (P) prevented degradation.

Because Cath-D is secreted by hormone-dependent breast cancer cell lines, we tested its concentration and its activity in the culture supernatants of the human cell lines MCF-7 and T47D grown in absence or presence of estradiol and EGF. Immunoblots with anti-Cath-D showed that Cath-D and the catalytically nonactive procathepsin D (Mr 31,000 and 45,000, respectively) were both present in culture supernatants (data not shown). Cath-D concentrations in the supernatants of MCF-7 cells were considerably higher than in those of T47D cells (Figure 2A). Therefore proteolysis experiments were performed on the supernatants of MCF-7 cells stimulated with estradiol and EGF. These supernatants completely degraded MIP-1β, whereas the simultaneous detection of cleaved and uncleaved SLC after 8 hours of incubation indicated only partial degradation (Figure 2B). As already observed in experiments using purified Cath-D, pepstatin A prevented processing of MIP-1β and SLC by culture supernatants. Conversely, SDF-1 was not cleaved by the culture supernatants even with incubations at pH 7. Taken together, the results demonstrate the involvement of

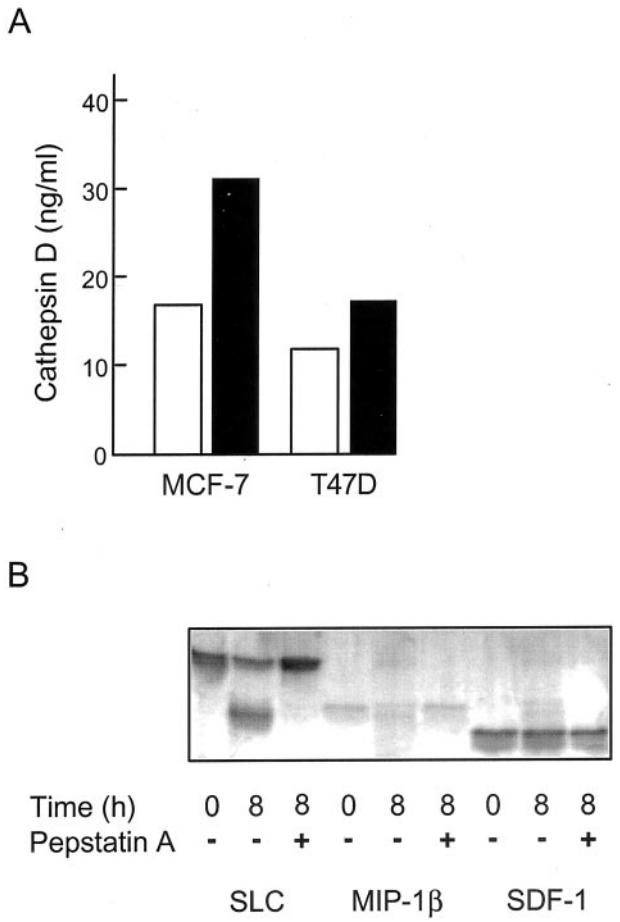

Figure 2. Cath-D in culture medium of human breast cancer cells. **A:** Cath-D concentration as assessed by ELISA in culture medium of MCF-7 and T47D cells stimulated for 72 hours with 5 nmol/L of estradiol and 125 ng/ml of EGF (**black bars**) or unstimulated (**white bars**). Values represent the mean of two experiments with duplicate determinations. **B:** Ten-μl culture medium of stimulated MCF-7 cells were incubated for 8 hours in the presence or absence of 1 μmol/L of pepstatin A with 5.0 μmol/L of SLC, MIP-1β, or SDF-1, and analyzed by SDS-PAGE.

Cath-D, but not of other proteases that might be present in cell culture supernatants, in processing these chemokines. Indeed, supernatants of MCF-7 cells contain significant concentrations of Cath-D as measured by ELISA; purified Cath-D and culture supernatants cleave specifically SLC and MIP-1β, but not SDF-1; the cleavage products of SLC after incubation either by purified Cath-D or cell supernatants have identical sizes; lastly, proteolysis is inhibited by pepstatin A in both conditions.

Identification of Cleavage Sites

Mass spectrometry analysis of the cleavage products of SLC demonstrated that Cath-D cleaves selectively the Leu[58] to Trp[59] bond (Figure 3) producing two fragments, the N-terminal SLC(1-58) with a mass of 6446 and the C-terminal SLC(59-111) with a mass of 5816. Both cleavage products were purified by reverse phase column chromatography and tested for biological activity using calcium mobilization assays in CCR7-transfected mouse

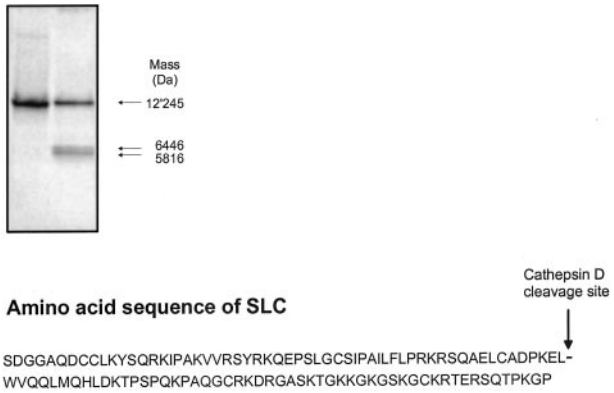

Figure 3. Characterization of SLC cleavage. SLC was incubated with purified Cath-D for 4 hours and analyzed by SDS-PAGE. Mass spectrometry analysis of the products yielded two peptides of 6446 d and 5816 d, respectively, and determined the cleavage site between Leu[58] and Trp[59].

pre-B cells. Neither fragment showed a functional effect (data not shown).

MIP-1α and MIP-1β were completely degraded by Cath-D and therefore not accessible for cleavage site determination. To circumvent this problem, a series of hybrid chemokines with N-terminal motifs of monocyte chemoattractant protein (MCP)-3 (CCL7), a chemokine that is not cleaved by Cath-D (Table 1), and variable lengths of MIP-1β sequences at the C-terminus were constructed (Figure 4). These molecules do not, or only very weakly bind to either the MCP-3 receptor (CCR2) or the MIP-1β receptor (CCR5) (I. Clark-Lewis, unpublished results). The hybrids were not completely degraded but yielded a cleavage product with slightly higher mobility on SDS-PAGE, and running at the same position where a transitional degradation product of MIP-1β could be detected. The cleavage products of MCP3H6 and MCP3H10 were further analyzed by mass spectrometry. In 80 to 90% of both constructs, the eight C-terminal

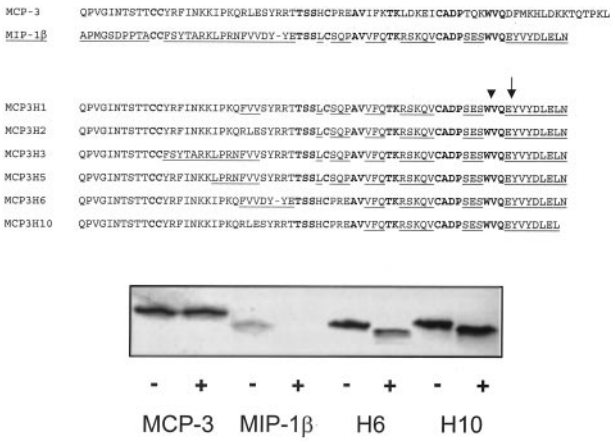

Figure 4. Characterization of MIP-1β cleavage site by using various MCP-3/MIP-1β hybrids. Five μmol/L of MCP-3/MIP-1β hybrids were incubated for 90 minutes with Cath-D (+) or left untreated (−), and the products analyzed by SDS-PAGE and Coomassie blue staining. Mass spectrometry analysis of the proteolytic products of MCP3H6 (H6) and MCP3H10 (H10) indicated cleavage sites between Glu[61] and Tyr[62] (**arrow**, 80% of H6 and 90% of H10), or between Trp[58] and Val[59] (**arrowhead**, 20% of H6 and 10% of H10). **Normal letters**, MCP-3-sequence; **underlined letters**, MIP-1β-sequence; **bold letters**, homologies (at least two amino acids) for both chemokines.

amino acids (YVYDLELN) were missing, indicating that cleavage took place between Glu^{61} and Tyr^{62}. Cleavage was not uniform as demonstrated by finding 10 to 20% of the molecules cut between Trp^{58} and Val^{59}, ie, cleaving off 11 C-terminal amino acids (VQEYVYDLELN). Thus, it can be assumed that processing of MIP-1β would also start by cleaving off amino acids at the C-terminal region, and that most likely a different folding of the MCP-3/MIP-1β hybrids prevents further degradation. Because MIP-1α and MIP-1β were completely degraded by Cath-D, and the hybrids do not bind to the CCR5 receptor, no functional assays were performed with the truncated hybrids.

MIP-1α, MIP-1β, SLC, and Cath-D Genes Are Expressed in Breast Cancer

To further characterize the biological significance of chemokine cleavage by Cath-D *in vivo*, we tested mRNA expression of all three chemokines and of Cath-D in serial sections of breast cancer specimens obtained from 10 patients. MIP-1α and MIP-1β were found in the stromal compartment of breast cancers, mainly in areas with conspicuous leukocytic infiltrates (Figure 5). The cellular sources of both chemokines could not be determined with certitude. However, based on the morphology and on immunohistochemical stainings of serial sections (data not shown), both chemokines are most likely produced by macrophages. MIP-1α transcripts were also occasionally found in cells with elongated nuclei, probably fibroblasts, whereas MIP-1β mRNA was occasionally visible in cancer cells. In contrast, SLC transcripts were detectable only in endothelial cells of capillaries and venules within the cancer stroma and in close proximity to leukocytic infiltrates (Figure 5). Cath-D mRNA was widely detectable in the stroma of breast cancers (Figure 6), including tissue compartments showing chemokine expression in serial sections and inflammatory infiltrates consisting mainly of macrophages and T cells. Based on the morphology and on immunohistochemical stainings, macrophages most likely account for a relevant cellular source of Cath-D. Occasionally, the transcripts could be observed in cancer cells (Figure 6), and possibly in fibroblasts.

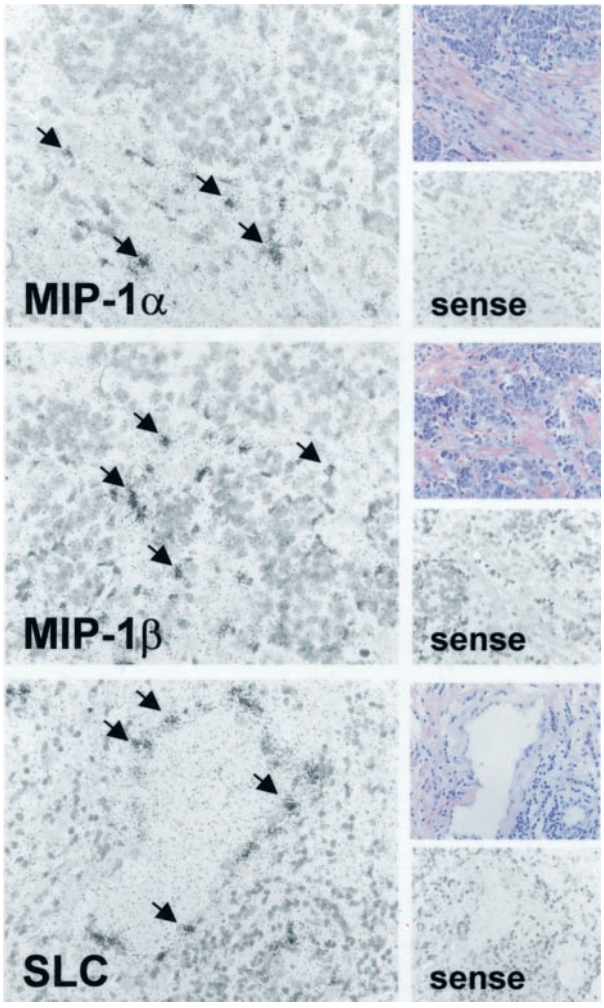

Figure 5. Chemokine mRNA expression in human breast cancers. **Left**: Expression of MIP-1α, MIP-1β, and SLC transcripts in invasive ductal carcinoma. MIP-1α and MIP-1β transcripts are mainly expressed in the stromal compartment. Morphology, study of serial sections, and immunohistochemical stainings (data not shown) indicate that macrophages most likely represent the major cellular sources for both chemokines. SLC mRNA is expressed in endothelial cells of venules surrounded by leukocytic infiltrates at the cancer border. **Right**: H&E-stained serial sections and sense hybridizations are shown for each chemokine.

Breast Cancer Cell Lines Do Not Release Cath-D in Response to a Subset of Chemokines

Chemokine and Cath-D gene expression occurs *in vivo* synchronously and in the same tissue compartments of breast cancers. It is conceivable that, to facilitate cell migration through the extracellular matrix, chemokines may directly affect the release of Cath-D. We therefore analyzed culture supernatants and cell lysates of MCF-7 and T47D cell lines by ELISA for Cath-D production in response to various chemokines. SLC, SDF-1, and IL-8 were selected because of the reported expression of the corresponding receptors (CCR7, CXCR4, and CXCR2, respectively) on breast cancer cell lines.[5] After 24 hours of incubation of both cell lines with the selected chemokines, no changes in Cath-D concentrations were observed, both in presence or absence of estradiol and EGF.

Discussion

Several reports document a role for chemokines in controlling cancer progression. Initially, chemokines have been recognized to have a direct effect on tumor growth by recruiting tumor-associated macrophages that may release tumor-stimulating cytokines and angiogenic factors,[4,17] or by a direct regulation of tumor angiogenesis.[18–20] Furthermore, recent reports highlight the roles of chemokines in modulating immune responses directed against the tumor through the recruitment of effector T cells, natural killer cells, and tumor-infiltrating dendritic cells (DCs).[4,21] Lastly, it became evident that chemo-

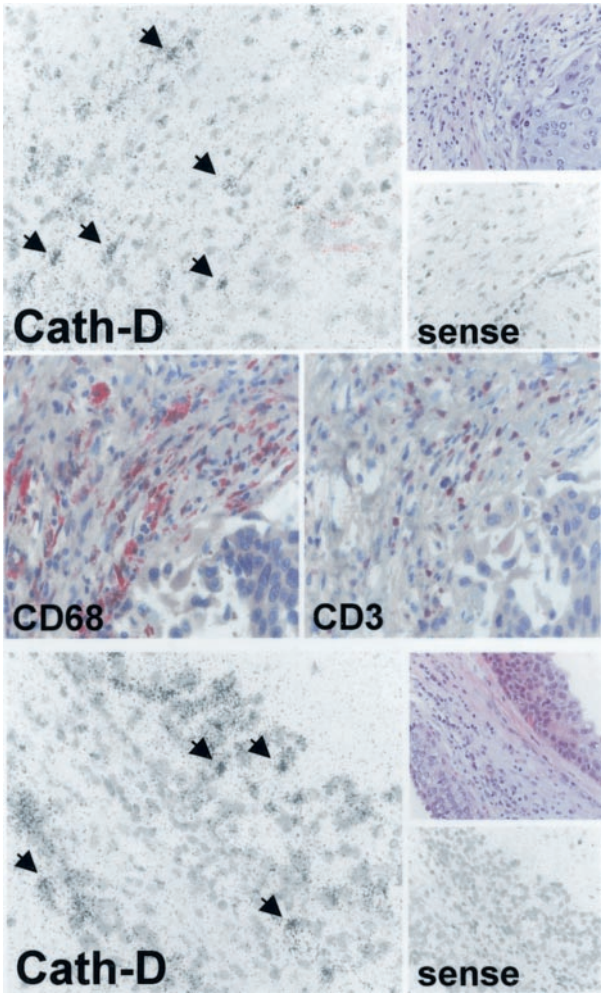

Figure 6. Cath-D mRNA expression in human breast cancers. **Top left**: Expression of Cath-D in the stromal compartment of invasive ductal carcinoma with leukocytic infiltrates consisting mainly of macrophages (CD68+, **middle left**) and T cells (CD3+, **middle right**) identified in adjacent sections. Morphology, study of serial sections, and immunohistochemical stainings indicate that macrophages and possibly fibroblasts account for the cellular sources of Cath-D. Some positive cells are highlighted by **arrows**. **Bottom left**: Expression of Cath-D transcripts in cancer cells (partially highlighted by **arrows**). H&E-stained serial sections and sense hybridizations are shown on the **right** (**top** and **bottom**).

kines may directly affect the motility of cancer cells in primary tumors or even orchestrate the migration of metastatic tumor cells to secondary organs.[5] In this report we show for the first time that Cath-D selectively degrades three chemokines, ie, MIP-1α, MIP-1β, and SLC that are expressed in primary breast cancers. These findings suggest that chemokine inactivation through degradation and clearance may represent an important regulatory mechanism in the complex cross-talk between cancer cells, tumor-associated leukocytes, and extracellular matrix components.

Proteolysis of SLC generated two biologically inactive peptides, but it was not completed even after overnight incubation with Cath-D. Complete cleavage of SLC *in vitro* may be prevented by complex formation between the molecules. Conversely, MIP-1α and MIP-1β were rapidly and completely degraded by Cath-D. Analysis of MCP-3/MIP-1β chimeric constructs showed that the initial Cath-D cleavage site is located on the C-terminal helix containing MIP-1β motifs. The partial degradation in MCP-3/MIP-1β hybrids, in contrast to the total degradation of MIP-1β, suggests that the primary amino acid sequence, but most likely also the tertiary structure, determines the specificity of Cath-D cleavage.

In our studies, the most efficient Cath-D cleavage was observed at pH 4 whereas at greater than pH 5.5 no proteolytic activity could be detected. These findings are consistent with previous reports demonstrating that acidic pH is required for Cath-D activity *in vitro*.[22,23] A pH lower than 5.5 may be rarely found in the extracellular environment of normal tissues. However, it is well known that the extracellular pH of tumors is acidic.[24] Most likely, multiple pathways contribute to an acidic microenvironment pH *in vivo*. For instance, previous studies indicate that breast cancer cells and macrophages within cancerous tissue have a high potential to liberate protons into the extracellular milieu through the proton pump of the vacuolar H^+-ATPase at the plasma membrane.[25] Furthermore, anoxia, a characteristic of many cancers, may also promote an acidic environment in tumors.[26] Lastly the β subunit of mitochondrial ATP synthase has been detected on the surface of cancer cells and may thus contribute to maintain an acidic extracellular pH through the production of extracellular ATP.[27] The significance of chemokine cleavage *in vivo* would ideally be tested through the demonstration of specific degradation products. However, the degradation products of MIP-1α and MIP-1β most likely consist of short oligopeptides or even single amino acids that, even if detectable in tissue samples, cannot be ascribed to a specific protein. Conversely, the SLC degradation products formed *in vitro* consist of two stable peptides. Unfortunately, no specific antibodies to recognize these peptides in tissue are available. It is also conceivable that these two peptides are further processed by other proteases *in vivo*. An additional point of debate about the role of Cath-D *in vivo* is the possible protection of chemokines by the presence of protease inhibitors in tissue. Pepstatin A, used in our *in vitro* experiment, is a bacterial product and it is not present *in vivo*. The hypothetical presence of protease inhibitors, however, would not cancel the significance of our findings but rather add another regulatory component affecting the complex chemokine-protease interactions.

The findings of the present study may have important functional implications in the biology of breast cancers. Indeed, chemokine cleavage by Cath-D can affect tumor progression at several levels. MIP-1β, as well as MIP-1α, are thought to participate in the recruitment of tumor-associated macrophages and T lymphocytes through binding to their receptors, ie, CCR5 and CCR5 or CCR1, respectively. Thus, it is conceivable that the release of Cath-D by tumor stromal cells, such as macrophages and fibroblasts, represents a significant autocrine and paracrine regulatory loop able to attenuate cell migration events through the disruption of a chemokine gradient in the extracellular matrix. Further, MIP-1α and MIP-1β may be implicated in regulatory pathways affecting the migration into tumor tissues of immature DCs that express the CCR1 and CCR5 receptors as well as others.[28] Tumor-

associated DCs play a key role in initiating a tumoricidal immune response at the primary sites[21] where, after exposure to neoplastic cells or tumor stroma, they convert to mature DCs and up-regulate the expression of the SLC receptor CCR7.[29] Antigen-loaded mature DCs can then promote an anti-tumor effect by migrating to secondary lymphoid organs.[30] In this context, the demonstration of SLC cleavage by Cath-D, and the detection of SLC transcripts in endothelial cells of tumor capillaries and venules, appear particularly interesting. In fact it can be hypothesized that Cath-D cleavage of SLC within primary tumor sites may perturb the migration of mature DCs to secondary lymphoid organs, representing therefore an escape mechanism of the anti-tumoral immune response. Alternatively, or in addition to the putative effects on tumor-associated leukocytes and DCs, it is also possible that degradation of chemokines by Cath-D directly affects the invasiveness and motility of breast cancer cells that respond to SLC, MIP-1α, and MIP-1β *in vitro*.[5,31]

Several chemokines among those not cleaved by Cath-D, such as RANTES (regulated on activation normal T-expressed and secreted, CCL5), MCP-1 (CCL2), SDF-1, and IL-8 are highly expressed in breast cancers.[5,32–35] These chemokines, however, could be degraded by other proteases expressed in breast cancers. In particular, it has been recently demonstrated that SDF-1, which is thought to promote motility of cancer cells, is specifically cleaved and inactivated by a panel of matrix metalloproteinases,[7] by cathepsin G,[9] and by leukocyte elastase.[8] Further, the inflammatory effect of other chemokines, eg, MCP-3, can be blocked by degradation through gelatinase A.[6] Finally, it is also possible that chemokine degradation by proteases generates truncated products that are biologically more potent. For instance, proteinases secreted by activated neutrophils potentiate IL-8 by amino-terminal processing, whereas they degrade other chemokines, such as growth related oncogene-α (CXCL1) and platelet factor-4 (CXCL4).[36,37] Collectively, these data highlight the complexity of the chemokine-protease network regulating cancer progression.

In the present studies we observed *de novo* synthesis of Cath-D in macrophages and possibly fibroblasts within the stroma of breast cancers, and occasionally in breast cancer cells. In estrogen receptor-positive breast cancer cells, Cath-D gene transcription is increased by estrogen and growth factors, whereas in estrogen-negative cancer cells it is constitutively expressed by an unknown mechanism.[2] It is conceivable that chemokines directly affect the release of proteases by cancer cells or by stromal cells to facilitate cell migration through basal membranes and the extracellular matrix. Along this line of thought, recent studies indicate that a subset of chemokines, such as MCP-1, MIP-1α, RANTES, and SDF-1 may affect the release of metalloproteinases in the extracellular milieu, and that in human fibroblasts and monocytes the enhancement of protease synthesis is related to a synergistic effect of endogenous IL-1α or tumor necrosis factor-α.[10,11,38,39] Although in the present studies, we did not observe Cath-D secretion on exposure of breast cancer cell lines and primary breast fibroblasts (data not shown) to a chemokine subset, further investigations are clearly necessary before excluding the existence of such autocrine or paracrine regulatory loops in *vivo*.

In conclusion, this study demonstrates that Cath-D, which is in general highly expressed and probably has prognostic implications in breast carcinomas, specifically degrades a subset of chemokines expressed in breast cancers. These results implicate novel regulatory pathophysiological mechanisms affecting the extracellular microenvironment. Further characterization of degradation by proteases *in vivo* may eventually lead to better understanding of the molecular basis of cancer invasion and metastasis.

Acknowledgments

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